REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
01-11-2013	Final Report	17-Sep-2012 - 16-Sep-2013

4. TITLE AND SUBTITLE
Competition for Shared Resources in the Cellular Chassis: Impact on Synthetic Circuits

5a. CONTRACT NUMBER
W911NF-12-1-0540

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHORS
PI: Domitilla Del Vecchio, Collaborator: Ron Weiss

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
Massachusetts Institute of Technology (MIT
77 Massachusetts Ave.
NE18-901
Cambridge, MA 02139 -4307

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS (ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
62621-LS-DRP.4

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
Protein overexpression leads to growth inhibition and decreased expression of native proteins. This effect is mostly due to competition for RNAP and ribosomes. This competition affects also synthetic biology circuits, which may not behave as expected. The aims of this project are to: (1) characterize/quantify the effect of competition for RNAPs and ribosomes on synthetic genetic circuits; (2) determine the extent to which RNAPs or ribosomes are limiting; (3) develop models that predict the change of behavior of circuits due to competition; (4) demonstrate the predictions on the design of a synthetic cascade. We

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Domitilla Del Vecchio
UU	UU	UU	UU		19b. TELEPHONE NUMBER 617-452-2275

Report Title

Competition for Shared Resources in the Cellular Chassis: Impact on Synthetic Circuits

ABSTRACT

Protein overexpression leads to growth inhibition and decreased expression of
native proteins. This effect is mostly due to competition for RNAP and ribosomes. This competition affects also synthetic biology circuits, which may not behave as expected. The aims of this project are to: (1) characterize/quantify the effect of competition for RNAPs and ribosomes on synthetic genetic circuits; (2) determine the extent to which RNAPs or ribosomes are limiting; (3) develop models that predict the change of behavior of circuits due to competition; (4) demonstrate the predictions on the design of a synthetic cascade. We have constructed a synthetic circuit in E. coli and characterized through theory and experiments the effect of competition on the expression of a constitutively expressed gene when a different inducible gene is activated. For this system, we have also developed predictive models, both analytical and computational. Our finding quantifies the extent of competition and provides initial tools to predict this effect. Our experimental results and
models will enable design methods for next-generation circuits that are robust to competition (to be investigated later). This ability will be critical for constructing large circuits reliably.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Paper

TOTAL:

Patents Submitted

Patents Awarded

Awards

Semi-plenary lecture at NOLCOS in September 2013

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Andras Gyorgy	1.00	
Jose Jimenez	1.00	
FTE Equivalent:	**2.00**	
Total Number:	**2**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Jose Jimenez	1.00
FTE Equivalent:	**1.00**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

Technology Transfer

TITLE: Competition for Shared Resources in the Cellular Chassis: Impact on Synthetic Circuits

Grant number: W911NF-12-1-0540

Final Progress Report, October 24, 2013

Period covered: September 16-October 17 2013

PI: Domitilla Del Vecchio

Collaborator: Ron Weiss

Institution: MIT

Contact: ddv@mit.edu

Abstract: Protein overexpression leads to growth inhibition and decreased expression of native proteins. This effect is mostly due to competition for RNAP and ribosomes. This competition affects also synthetic biology circuits, which may not behave as expected. The aims of this project are to: (1) characterize/quantify the effect of competition for RNAPs and ribosomes on synthetic genetic circuits; (2) determine the extent to which RNAPs or ribosomes are limiting; (3) develop models that predict the change of behavior of circuits due to competition; (4) demonstrate the predictions on the design of a synthetic cascade. We have constructed a synthetic circuit in *E. coli* and characterized through theory and experiments the effect of competition on the expression of a constitutively expressed gene when a different inducible gene is activated. For this system, we have also developed predictive models, both analytical and computational. Our finding quantifies the extent of competition and provides initial tools to predict this effect. Our experimental results and models will enable design methods for next-generation circuits that are robust to competition (to be investigated later). This ability will be critical for constructing large circuits reliably.

Papers Published: --

Presentations: --

Manuscripts: --*In preparation*.

Books: --*In preparation (with Richard Murray)*

Honors and Awards: --*Semi-plenary lecture at NOLCOS in September 2013*

Personnel supported: Andras Gyorgy-PhD student (100%); Jose Jimenez-Postdoc (100%)

Scientific Progress and Accomplishments since September 18 2013:

In this final report we will present the major achievements obtained during the period of duration of the grant. The findings here presented are currently being summarized in a manuscript that will be completed and submitted for publication in the following weeks.

1. A framework to study competition for cellular resources in *Escherichia coli*

One of the priorities of the project was to build a system to quantify the extent of the competition of different genes for the same cellular resources. We generated a synthetic circuit (MBP-1.0) that allows us to study the simultaneous expression of two reporter genes encoding a green (GFP) and a red (RFP) fluorescent protein. The circuit is contained in a plasmid with a tunable copy number. One of the reporters, GFP, is expressed constitutively whereas the other is under the control of the transcriptional activator LuxR. When the input of LuxR, the inducer acyl homoserine lactone (AHL), is added to the culture medium, LuxR binds to the promoter *Plux* and RFP is produced using resources that were initially allocated into the GFP synthesis. As a consequence, the amount of GFP generated is decreased (Fig. 1A).

We monitored in a flow cytometer the production of both reporters during exponential cell growth on glucose 0.4% as the sole carbon source. The data shows that as the concentration of RFP increases, the concentration of GFP decreases by up to a 69.4% for maximal AHL (Fig. 1B, left panel). This observation correlates well with numerical simulations (Fig. 1B, right panel) of a system of ordinary differential equations that accounts for the conservation of ribosomes and RNA polymerase along with the production and degradation of mRNAs and proteins. The parameters used in the model were obtained from the literature.

We considered several alternative reasons to competition for cellular resources that could explain our observations. All of them were discarded experimentally being the controls shown in Fig. 1C the most representative. Toxic effects of the inducer and RFP production were ruled out using a control that does not contain RFP (MBP-dRFP) and a control that produces a protein different from RFP (MBP-gapA), respectively. GapA encodes for the glyceraldehyde dehydrogenase from *E. coli* and it is very abundant in the bacterial cytoplasm. The control MBP-dRFP does not display an effect on GFP whereas the control MBP-gapA shows a decrease in GFP very similar to the original in the circuit MBP-1.0 (Fig. 1D). As a conclusion of the experiment we demonstrate that it is not the especific production of RFP but the process of production of a protein what is decreasing the synthesis of GFP, due to the competition for limited cellular resources.

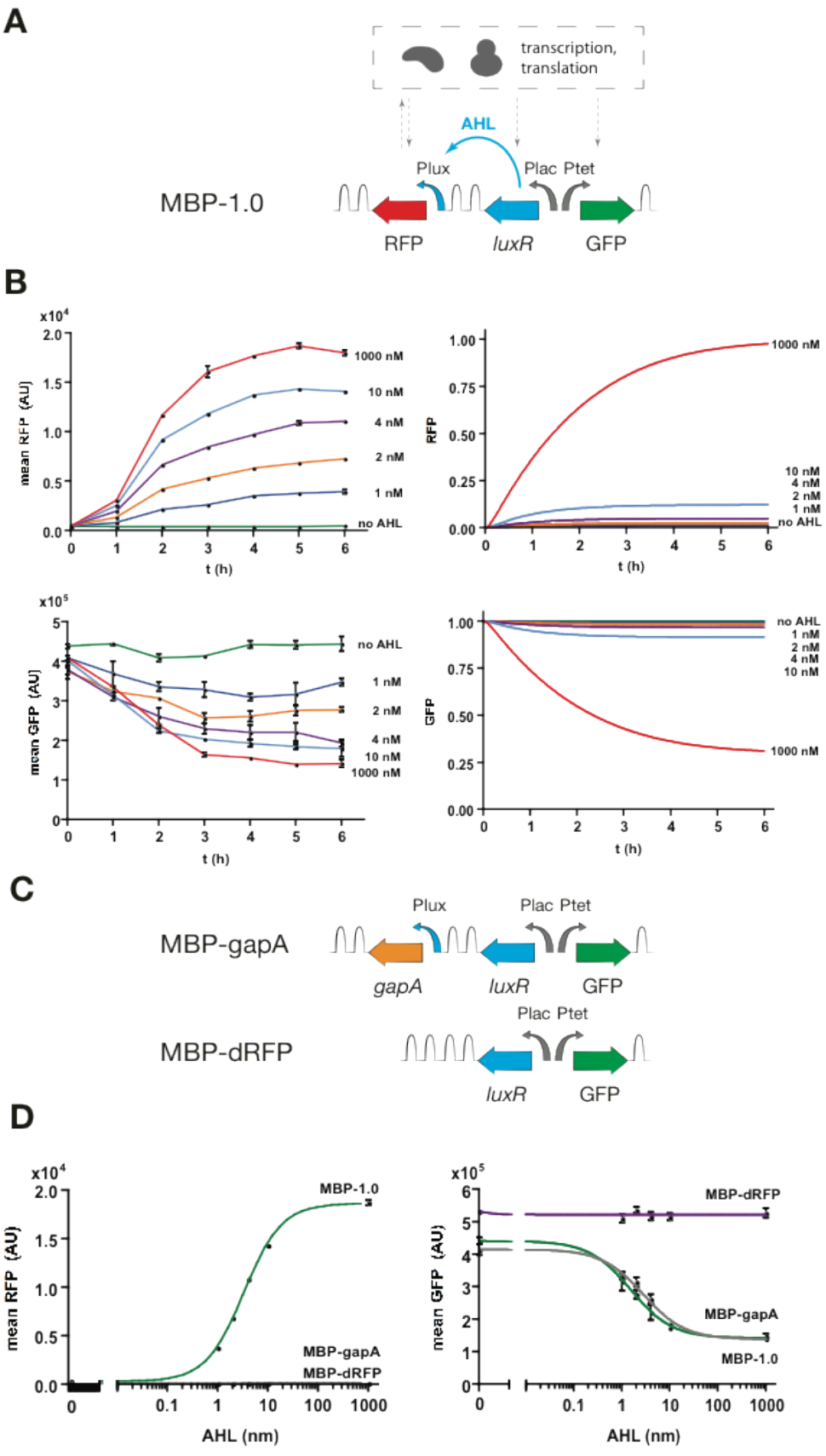
A
transcription,
translation
AHL
Plux
Plac Ptet
MBP-1.0
RFP
luxR
GFP
B
mean RFP (AU)
t (h)
1000 nM
10 nM
4 nM
2 nM
1 nM
no AHL
RFP
mean GFP (AU)
GFP
C
MBP-gapA
gapA
MBP-dRFP
D
MBP-1.0
MBP-gapA
MBP-dRFP
AHL (nm)

Fig. 1. Schematic representation of the circuits used to study competition (A). GFP is constitutively expressed and RFP is under the control of activator LuxR and input AHL. Curved arrows, promoters; hairpins, terminators. RFP (upper row) and GFP (lower row) expression dynamics (B). Each colored line corresponds to a concentration of inducer AHL in the experimental results (left panel) and simulations (right panel). (C) Control circuit MBP-dRFP does not contain RFP whereas in MBP-gapA the RFP gene has been replaced by the glyceraldehyde dehydrogenase encoding gene (gapA) from *E. coli*. (D) Dose response plots for circuit MBP-1.0 and the controls MBP-dRFP and MBP-gapA in the steady state. Left panel shows GFP and right panel shows RFP (note that the controls do not produce RFP). All plots represent mean values and standard deviations of populations analyzed by flow cytometry in three independent experiments.

2. Influence of RBS strength and copy number

In order to determine the relative contribution of RNA polymerase depletion and ribosome depletion to the GFP decrease, we created a set of circuits with progressively weaker RBS strength for the RFP gene. The weakest RBS extents minimal demand for ribosomes by RFP mRNA, providing a way to assess the contribution of depletion RNA polymerase depletion on the reduction of GFP. We used a set of RBS sequences that range from very strong (MBP-1.0) to very weak undetectable translation of RFP (MBP-0.006). The dose response curves show that weaker RBS strengths have reduced effects on GFP, with no appreciable effect in the case of MBP-0.006 (Fig. 2A). Numerical simulations of the ODEs are consistent with the experimental data (Fig. 2B). This result supports the hypothesis that the pool of RNA polymerase is not appreciably reduced due to transcription of the RFP gene and hence that ribosome depletion is the contributor to the 68.4% reduction of GFP observed in Fig 1.

Another way to reduce the coupling between RFP and GFP expression is to decrease the plasmid copy number. We tested two lower plasmid copy numbers to analyze the extent to which the coupling could be reduced. Instead of replacing the origin of replication, which may in turn generate artifacts due to the involvement of different replication machinery for each of the origins, we used the DIAL strains (Kittleson *et al*, J. Biol. Eng. 2011, 5:10). For these experiments we always used the original J92001 construct but changed the host, to tune the number of copies of the circuit. The nominal values in the exponential phase of the copies tested were 64±2 (J, high), 30±14 (H, mid) and 4±1 (E, low). The results show that the extent of competition depends on the number of copies of the plasmid: it is reduced to 55% with medium plasmid copy number and to a 29% with the lowest copy number tested (Figs. 2B). This observation is in good agreement with the model prediction.

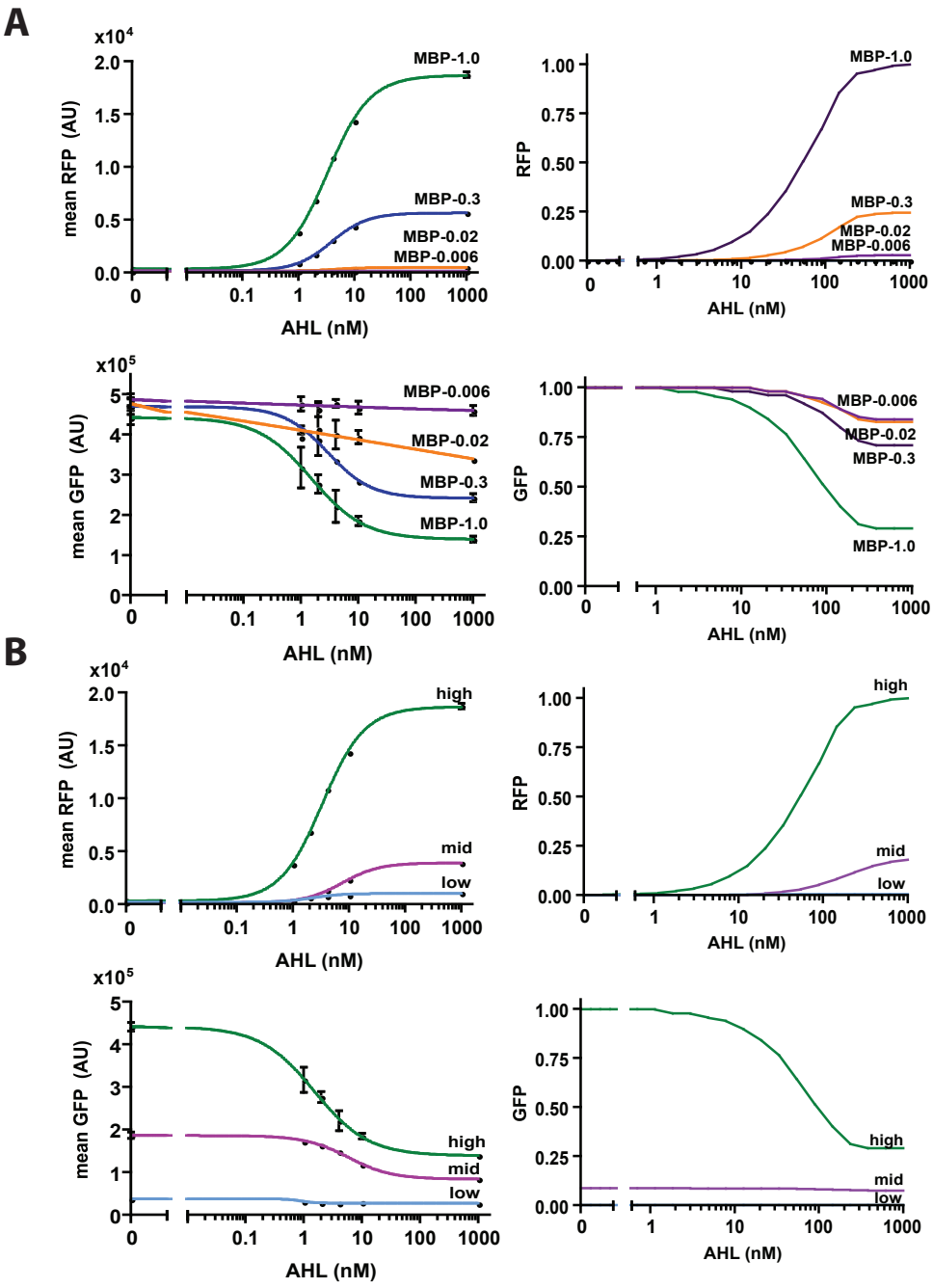

Fig. 2. AHL dose response plots as a function of RFP's RBS strength and plasmid copy number. Left and right columns show, respectively, experimental and computational results for both RFP (odd rows) and GFP (even rows). (A) RBS strength effects. The numbers following the suffix MBP- indicate the relative strength of the RBS for RFP compared to MBP-1.0. (B) Plasmid copy number. The plasmid MBP-1.0 was tested in the DIAL hosts JTK60 J (61-65 copies), H (16-44 copies) and E (3-5 copies). These copy numbers lead to 68.4%, 28%, and 29% change in GFP, respectively. Experimental values depict mean and standard deviation of the populations for three independent steady state experiments.

3. Analytical model and linear relationships between reporters concentration

To discover how key parameters control the extent of coupling we constructed an analytical model that predicts how the concentration of one protein (GFP, for example) is affected by induction of a different protein (RFP, for example) due to sharing RNA polymerase and ribosomes. This model is constructed starting from the computational model used for the simulation results and making some approximations that are supported by the data. Specifically, these approximations assume that the concentration of free RNA polymerase and of free ribosomes is sufficiently smaller compared to the respective dissociation constants with DNA and RBS, respectively. The opposite limit cases when some dissociation constant is much smaller than the free amount of RNA polymerase or ribosome are not supported by data.

Based on this model, the concentration of protein P_i (i=1,2) is given by

$$P_i = \frac{\varepsilon_i D_i \alpha_i}{AB + \sum_{i=1}^{2} D_i[\varepsilon_i (A/K_i) + (x_T/K_i)(m_i/\tilde{K}_i)]} \Big/ x_T y_T / K_i \tilde{K}_i$$

in which A and B are constants independent of the synthetic circuit, x_T and y_T are the available concentrations of RNA polymerase and ribosomes, K_i is the dissociation constant of RNA polymerase from the promoter of gene i, $\tilde{K}_i$ is the dissociation constant of ribosomes from the RBS of gene i (inversely proportional to the RBS strength), D_i is the DNA copy number, α_i is a constant, m_i is the amount of mRNA per gene copy, and ε_i is a number between 0 and 1 and represents the level of induction of the promoter (See SI for more details). This expression quantitatively predicts that as protein 1 is induced, protein 2's concentration is reduced and the extent of reduction depends on the various parameters as indicated by the formula. Specifically, as $\tilde{K}_1$ is increased (weaker RBS strength for gene 1), the reduction in protein 2's concentration is less severe. Similarly, as the DNA amount is decreased, less reduction of protein 2's concentration is observed, but this is to the expense of a lower value of the concentration of protein 1. By This formula, one can predict that a linear relationship between P_1 and P_2 holds:

$$P_2 = -aP_1 + q$$

in which $a>0$, that is, the concentration of the two proteins is constrained on a linear manifold when protein 1 is induced. The slope of the line a increases as the dissociation constant $\tilde{K}_1$ is decreased, that is, the RBS for gene 1 is weakened. The intercept q, instead, stays constant as the RBS strength of gene 1 is changed (Fig 1A).

The analytical model can be used to study the case in which there are two inducible modules (Fig. 3B). Specifically, the linear relationship for protein production can be applied to both modules, rendering two lines with different slopes and offsets. In the absence of competition, the amount of proteins produced by the circuit could lay anywhere in the surface delimited by maximum production of each protein (Fig. 3B, left panel). However, because of the competition for RNAP and ribosomes, the achievable region for protein production is limited to the area comprised by the linear manifolds of both modules and the axes (Fig. 3B, right panel).

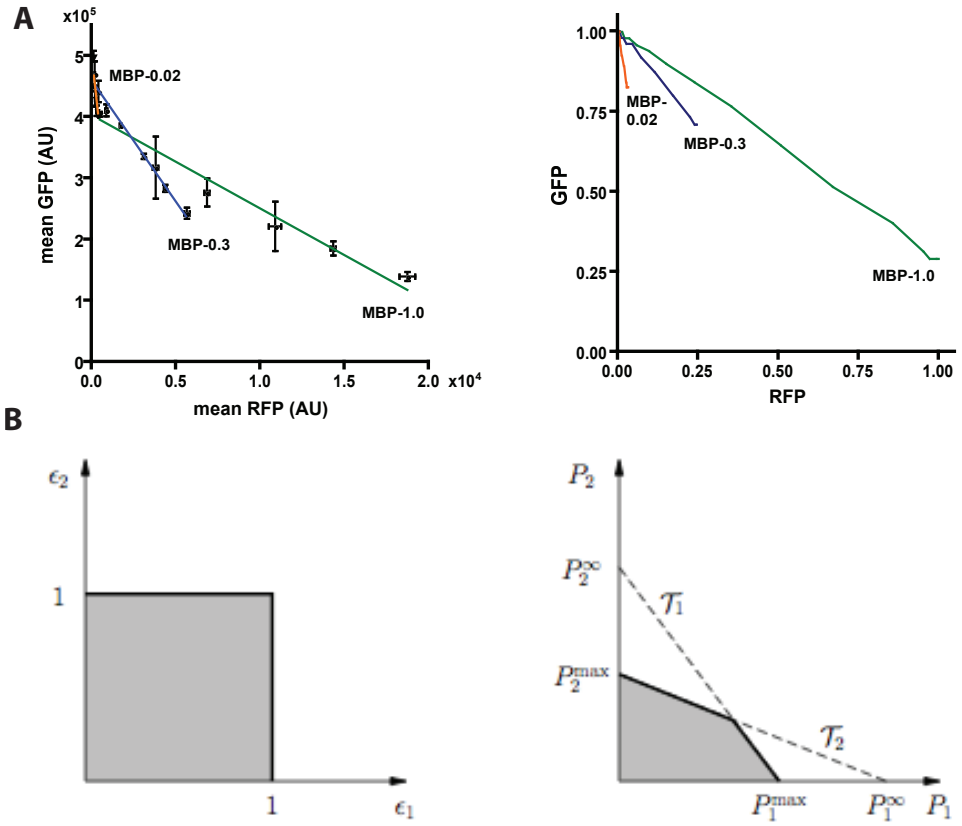

Fig. 3. (A) Linear relationships between GFP and RFP production. This plot is a summary of the information presented in the rest of the figures and compares the emission of GFP and RFP controlled by the various RBSs. Experimental results (left column in A and B) are compared to computational simulations (right column in A and B). (B) Regions allowed for protein production in the case of using two inducible modules. The left panel represents the case where there is no competition between the genes and each of the proteins can be produced up to its maximum rate. The right panel shows the region allowed for protein production when there is competition for cellular resources, and the allowable region constrained by the two linear manifolds.

3. A test case circuit affected by competition: A cascade of transcriptional activators

In the previous results we have studied the case where two unconnected modules are coupled due to the competition for the same resources. Solving that problem allows us to We have just finished building a new synthetic circuit that describes the case in which the genes competing for the same cellular resources are also transcriptionally linked. The circuit is composed by a cascade of two transcriptional activators that end up producing a fluorescent reporter in the presence of the appropriate inducers (Fig. 4A). The circuit is triggered by acyl homoserine lactone (AHL) that binds to the regulator LuxR. LuxR in turn activates the transcription of the second regulator, NahR, which in response to its cognate inducer salicylate (SAL) binds to the promoter *Psal* and promotes the expression of GFP.

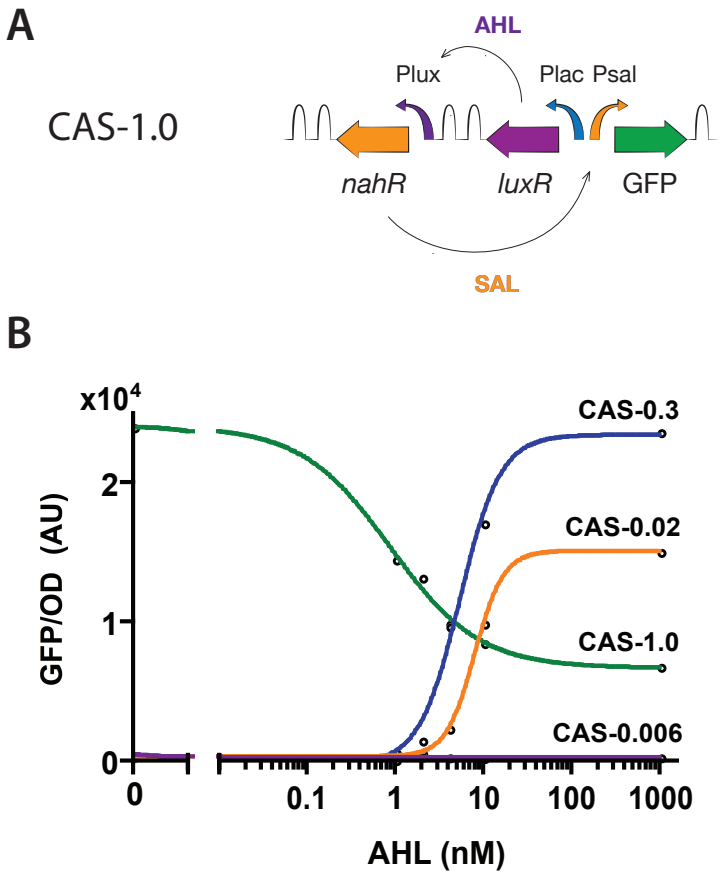

Fig. 4. (A) Schematic representation of the circuit encoding the cascade of transcriptional activators. LuxR is expressed constitutively and produces NahR in response to AHL. In the presence of SAL NahR triggers the expression of GFP. (B) Dose response plots as a function of AHL for different variants of the cascade where the RBS of *nahR* has been tuned. The concentration of SAL is 1 mM in all the experiments. A strong RBS for *nahR* (CAS-1.0) is very demanding for ribosomes and synthesis of GFP is decreased despite producing more molecules of the activator. Weaker versions of *nahR* RBS (CAS-0.3 and CAS-0.02) display lower competition and the cascade behaves as expected. GFP is not produced when the RBS of *nahR* is too weak (CAS-0.006) or there is no SAL in the culture medium (not shown).

We built the cascade and monitored the production of GFP as a function of the first inducer AHL when the second was kept always at a saturating concentration of 1 mM (Fig. 4B). Intuitively, an increased production of GFP should be expected when the concentration of the input AHL also increases. However, the experimental results show the opposite behavior and in turn less GFP is formed with increasing AHL. That observation can be explained due to the competition of the two genes, *nahR* and GFP for the same cellular resources. An excessive synthesis of NahR uses up all the resources and the production of GFP is decreased.

The expected behavior is nevertheless restored when using variants of the circuit with a weaker RBS for *nahR* (CAS-0.3 and CAS-0.06). In those experiments, the dose response plots show an increased production of GFP in response to increasing concentrations of AHL. In agreement with our previous results using unconnected modules, a similar output would be expected when reducing the copy number of the cascade.

It is worth noting that in the presence of saturating concentrations of SAL and in the absence of AHL the circuit with the strongest RBS for *nahR* produces a high amount of GFP (first experimental point in CAS-1.0; Fig. 4B). That amount of GFP is produced as a consequence of the basal transcription of *nahR* that may be originated because of an unspecific activation mediated by LuxR or an incomplete termination of the transcript originated from *Plac.* In any case, the result would be the same, a small amount of NahR molecules that can trigger GFP production in the absence of competition. We are currently trying to reduce this basal production using other circuit topologies to have a better understanding of the system.

Taken altogether, these results validate our framework to study competition of different genes for the same cellular resources. In this particular example, excessive production of one of the activators has a consequence the opposite of the desired behavior.

Current impact:

With the findings of this project, we now have the necessary knowledge to determine how circuits should be designed so that coupling effects due to unavoidable resource sharing are minimized. This will enable the creation of large circuits expressing many molecules that behave predictably once interacting in the cell environment.